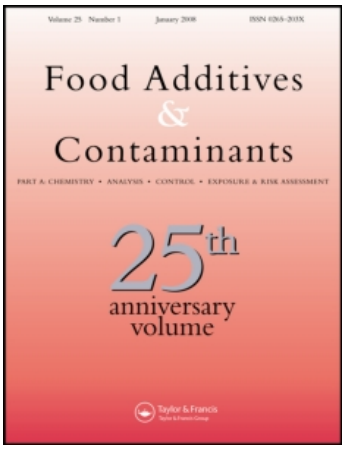

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Genomic analysis of Fusarium verticillioides

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Genomic analysis of *Fusarium verticillioides*

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Fusarium verticillioides (teleomorph *Gibberella moniliformis*) can be either an endophyte of maize, causing no visible disease, or a pathogen-causing disease of ears, stalks, roots and seedlings. At any stage, this fungus can synthesize fumonisins, a family of mycotoxins structurally similar to the sphingolipid sphinganine. Ingestion of fumonisin-contaminated maize has been associated with a number of animal diseases, including cancer in rodents, and exposure has been correlated with human oesophageal cancer in some regions of the world, and some evidence suggests that fumonisins are a risk factor for neural tube defects. A primary goal of the authors' laboratory is to eliminate fumonisin contamination of maize and maize products. Understanding how and why these toxins are made and the *F. verticillioides*–maize disease process will allow one to develop novel strategies to limit tissue destruction (rot) and fumonisin production. To meet this goal, genomic sequence data, expressed sequence tags (ESTs) and microarrays are being used to identify *F. verticillioides* genes involved in the biosynthesis of toxins and plant pathogenesis. This paper describes the current status of *F. verticillioides* genomic resources and three approaches being used to mine microarray data from a wild-type strain cultured in liquid fumonisin production medium for 12, 24, 48, 72, 96 and 120 h. Taken together, these approaches demonstrate the power of microarray technology to provide information on different biological processes.

Keywords: microarray; expressed sequence tags (ESTs); microbiology; mycology; fumonisins; mycotoxins – *Fusarium*; cereals and grain

Introduction

Fusarium verticillioides (teleomorph *Gibberella moniliformis*) is recognized as both a systemic endophyte and a pathogen of maize. As an endophyte, *F. verticillioides* prevents opportunistic saprotrophs such as *Aspergillus flavus* from spreading within the ear and rotting the seeds (Wicklow et al. 1988; Yates et al. 1997). In contrast, as a pathogen, *F. verticillioides* disease is associated with every life stage of the plant causing root rot, stalk rot, kernel or ear rot, seed rot, and seedling bight (Cook 1981; Munkvold 2003). Economic loss due to this fungus also stems from contamination of maize with fumonisins, a family of polyketide-derived mycotoxins. Ingestion of fumonisins is linked to a number of animal diseases, including cancer in laboratory rodents (Howard et al. 2001), and has been correlated with human oesophageal cancer in some regions of the world (Marasas 2001). Recent findings also suggest that fumonisin exposure increases the risk of neural tube defects, likely due to interference with the utilization of folic acid (Marasas et al. 2004; Gelineau-van Waes et al. 2005; Missmer et al. 2006). Fumonisins are structurally related to the free sphingoid base sphinganine and disrupt sphingolipid metabolism through the inhibition of the enzyme ceramide synthase (Merrill et al. 1993). In addition to fumonisins, *F. verticillioides* synthesizes a number of other fungal metabolites some of which, like the mycotoxin fusarin C, can also be found in maize (Desjardins and Proctor 2001).

The role fumonisins play in endophytic or pathogenic growth of *F. verticillioides* is poorly understood. Studies with maize seedlings are conflicting. Early work found that fumonisins likely play a role in seedling blight, but that they are not necessary to cause disease (Desjardins et al. 1995). Later work indicates that fumonisin is a phytotoxin responsible for disease symptoms on seedlings (Glenn et al. 2004; Williams et al. 2007). Studies with mature maize ears found that fumonisins are not required to cause maize ear rot or ear infection (Desjardins et al. 2002). Recent work has shown that the roots of maize seedling exposed to fumonisins have elevated levels of free sphingoid bases (Williams et al. 2006). In both plant and animal model systems intermediates in the ceramide biosynthetic pathway (e.g. sphingoid bases) are important second messengers in stress responses (Ng et al. 2001; Hannun and Obeid 2002; Chalfant and Spiegel 2005). The role,

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if any, of the increase in sphingoid bases in *F. verticillioides*–maize interaction remains to be determined.

Much of *F. verticillioides* research has centred on examining the biochemical and regulatory aspects of fumonisin biosynthesis. The major long-term goal of this research is to use this understanding to develop strategies that reduce the presence of fumonisins in maize in order to limit their harmful effects on human and animal health. The recent development of a variety of *Fusarium* genomic tools is having a dramatic impact on research programmes on toxin synthesis and *Fusarium*–plant endophytic or pathogenic interactions. This report will summarize *Fusarium* genomic tools and provide examples where they are being used to understand complex biological processes.

F. verticillioides genomic resources

F. verticillioides genomic resources include genetic maps, expressed sequence tag (EST) database, 8X genomic sequence and a predicted protein set for over 14,000 genes. The most extensive genetic map was generated from 636 biochemical, molecular and morphological markers and a mapping population of 121 meiotic progeny. The map consists of 12 linkage groups and spans 2188 cM (map units) with an average interval of 3.9 map units between markers (Xu and Leslie 1996; Jurgenson et al. 2002). The Broad Institute (Boston, MA, USA) generated nucleotide sequence data for 131 of the molecular markers (restriction fragment length polymorphisms, RFLPs) and matched them to genomic sequence effectively anchoring 99% of the genetic map. The *F. verticillioides* EST database consists of over 87,000 ESTs that have been compiled into the *F. verticillioides* Gene Index (FvGI) (Brown et al. 2005). The FvGI was a collaborative project between the USDA-ARS NCAUR Mycotoxin Research Unit and The Institute for Genomics Research (TIGR, Rockville, MD, USA; now a part of the J. Craig Venter Institute, JCVI) and is located at the Dana Farber Cancer Institute (DFCI, Boston, MA, USA) (Table 1). A majority of the ESTs were generated from nine different cDNA libraries and correspond to 11,119 unique sequences. Based on a comparison with the recently released genome sequence and annotation, we estimate that the FvGI represents approximately 78% of all *F. verticillioides* genes (see below).

The *F. verticillioides* genomic sequence was generated by a consortium of industrial and academic institutions and coordinated by the Broad Institute. The first half of the 8X total genome sequence was provided by Syngenta (Basel, Switzerland) and funding for the second half as well as assemble costs was provided by the National Research Initiative, of the US Department of Agriculture's Cooperative State Research Education and Extension Service. On 3 November 2006, the Broad Institute released the 8X genome sequence which covers over 41.7 Mb of DNA and spans 210 contigs in 36 supercontigs (GenBank accession no. AAIM02000000). The Broad Institute subsequently released predictions for a set of 14,179 genes based on EST, genomic sequence data, and various gene calling programs (Table 1).

Application of microarrays

Co-regulation

Now that approximately 14,000 genes have been identified in the *F. verticillioides* genome, one of the challenges facing *F. verticillioides* researchers is deciding which of the thousands of genes to characterize functionally. Functional studies of individual genes require significant time and effort and is recognized as the major bottleneck limiting fungal researchers efforts to developing new strategies to control pre-harvest mycotoxin contamination. One approach to narrow the pool/number of candidate genes for functional studies is to examine the relative expression of all genes using DNA microarrays. Differential expression of messenger RNA has been used extensively to identify genes or gene products involved in specific biological processes. NimbleGen Systems Inc. has developed an oligonucleotide-based array where each gene can be represented on the array by up to 12 pairs of oligos, tiled across the gene, where one oligo of a pair is a perfect match and the other contains a single nucleotide difference (reviewed in Aharoni and Vorst 2002). Our first generation *F. verticillioides* microarray chip

Table 1. *F. verticillioides* genomic resources on the internet.

Sequence	Organization	Reference	Website
Genetic Map	Kansas State University	Jurgenson et al. (2002)	http://www.broad.mit.edu/annotation/genome/fusarium_verticillioides/Home.html
ESTs (≥87,000)	The Institute for Genomics Research (TIGR)	Brown et al. (2005)	compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=f_verticill
Genomic	Broad Institute	Not applicable	http://www.broad.mit.edu/annotation/genome/fusarium_verticillioides/Home.html

was designed by NimbleGen based primarily on the FvGI, in collaboration with TIGR and the USDA. Each chip includes oligos that essentially represent all of the genes identified in the FvGI as well as most of the introns of fumonisin (*FUM*) biosynthetic genes.

The data analysis described herein was generated from a microarray experiment conducted with RNA extracted from *F. verticillioides* strain M-3125 cultured over a 5-day time course in liquid GYAM medium (Proctor et al. 1999). GYAM supports fumonisin production but only after several days of incubation. Samples of mycelium were taken at 12, 24, 48, 72, 96 and 120 h and total RNA from two biological replications were extracted using Trizol reagent (Invitrogen^{TM}) following established protocols (Brown et al. 2005). Samples were assayed for quality, frozen and shipped on dry ice to NimbleGen Systems (Madison, WI, USA) for analysis, hybridization, and data collection and visualized in Peoria using Acuity 4.0 software. As expected, *FUM* gene transcripts are minimal early, increase markedly after 48 h, and stay at high levels though 120 h (Proctor et al. 1999; Brown et al. 2006).

Although much progress has been made determining the function of genes in the fumonisin (*FUM*) biosynthetic gene cluster, relatively little is known about genes that regulate biosynthesis and that protect *F. verticillioides* from the harmful effects of fumonisins. It was reasoned that genes involved in these processes and not in the cluster may be expressed similarly to genes in the cluster. Precedence for co-expression of a gene(s) unlinked to a secondary metabolic biosynthetic gene cluster exists in other *Fusarium* species (Brown et al. 2003). In *F. graminearum*, the *TRI1* gene is required for the oxidation of carbon-8 on the trichothecene skeleton of deoxynivalenol (DON) and is co-expressed with other genes required for DON synthesis. This co-expression is likely due to specific DON transcription factor binding sites within the *TRI1* promoter sequence (Brown et al. 2003).

We began the microarray data analysis by examining the expression of the fumonisin biosynthetic gene *FUM1*, which encodes a polyketide synthase and is located in the *FUM* cluster. *FUM1* expression markedly increased from 7.7 ($\log_2$ scale) at 24 h to 13.3 at 72 h, thus the change (Δ) in expression or $\Delta\log_2$ was 5.6. This change in expression is consistent with previous Northern analysis of *FUM* genes (Proctor et al. 1999; Brown et al. 2006). Next, we identified a set of 66 sequence IDs in the microarray that exhibited changes in expression similar to *FUM1*, that is sequences that exhibit $\Delta\log_2$ values of 3.0–7.0. Of these sequences, 54 corresponded to genes in the *FUM* gene cluster. The other twelve sequence IDs correspond to eleven genes outside the cluster. BLASTX (Basic Local Alignment Tool) analysis of these eleven sequences against the National Center for Biotechnology Information (NCBI) database indicated that six shared significant similarity with previously characterized genes (Table 2). The predicted function of some of these co-expressed genes suggest a possible role in fumonisin biosynthesis. For example, TC30518 and TC31389 (were TC is a tentative consensus

Table 2. BLASTX analysis of sequences with pattern of expression to similar fumonisin biosynthetic genes.

Sequence ID[a]	$\Delta\log_2$[b]	TC/EST[c]	Putative function	*p*-value	Putative Fg homologue[d]
FUSA0001S00002460	4.2	FVJAV65TH	Unknown	n.a.	2×10^{-17} (FG00260.1)
FUSA0001S00007469	3.4	TC26172	Unknown	n.a.	1×10^{-35} (FG04283.1)
FUSA0001S00002813	3.7	FVNAV61TH	Unknown	n.a.	No
FUSA0001S00011496	4.5	TC30221	Unknown	n.a.	8×10^{-25} (FG02806.1)
FUSA0001S00009719	3.5	TC28433	Unknown	n.a.	3×10^{-148} (FG04664.1)
FUSA0001S00009324	4.2	TC28038	Dehydrogenase	1×10^{-134}	1×10^{-157} (FG04196.1)
FUSA0001S00009596	4.3	TC28310	Protein kinase	2×10^{-27}	8×10^{-46} (FG04804.1)
FUSA0001S00011284	3.0	TC30009	Sulfite oxidase	2×10^{-55}	0.0 (FG02880.1)
FUSA0001S00011788	4.7	TC30518	Transporter	5×10^{-78}	4×10^{-172} (FG00026.1)
FUSA0001S00011141	3.4	TC29864	Oligo-1,6-glucosidase	1×10^{-147}	2×10^{-142} (FG01235.1)
FUSA0001S00011142[e]		TC29865			
FUSA0001S00012656	3.5	TC31389	MFS transporter	5×10^{-35}	2×10^{-39} (FG03345.1)

Notes: [a]Sequence identification is the identification number randomly assigned to sets of twelve oligos that correspond to a single TC or EST sequence.
[b]$\Delta\log_2$ is the relative expression change at 72 vs. 24 h in liquid GYAM medium in the logarithmic base 2 ratio.
[c]TC/EST, tentative consensus sequence/singleton sequence in the *F. verticillioides* Gene Index.
[d]Possible *F. graminearum* homologue of the putative *F. verticillioides* putative gene based on BLASTX analysis. Numerical values are the most significant BLAST probability value to a putative *F. graminearum* gene followed by the genes Broad Institutes designation. No = no homologue.
[e]FUSA0001S00011141 and FUSA0001S00011142 refer to TC29864 and TC29865, respectively, which are alternative splice forms of each other. The average $\Delta\log_2$ value is presented.
n.a., Not applicable.

sequence) encode putative transport proteins and may play a role in transporting fumonisin from the fungus. Alternatively, the putative protein kinase encoded by TC28310 may play a role in regulation. Experiments are currently in progress to explore the possible role these genes play in fumonisin biosynthesis as well as the life cycle of the fungus.

Almost all eleven genes noted above may have homologues in the maize pathogen *Fusarium graminearum* (Table 2). The lone exception is the singleton FVNAV61TH. Analysis of *F. graminearum* genome sequence data indicates that all of the genes in the fumonisin gene cluster are absent. It is tempting to speculate that FVNAV61TH may play a role directly related to fumonisin biosynthesis while the roles played by the other co-regulated genes may be more general and affect multiple fungal processes.

Clustered co-regulation

Another strategy to identify genes that may play important roles in the *F. verticillioides*–maize interaction, is to examine the expression pattern of genes located adjacent to genes that are likely to play a role in the synthesis of secondary metabolites. It is possible that polyketides other that fumonisins contribute to the disease process. Polyketide synthases (PKSs) are a large, diverse family of multifunctional proteins that are responsible for the synthesis of polyketides. The wide diversity of PKSs gives rise to a wide diversity of polyketides, some of which pose health and economic risks (e.g. fumonisins and aflatoxins in agriculture) while others are a boon (e.g. lovastatin in the pharmaceutical industry and anthraquinones in the dyestuffs industry). Previous phylogenetic analysis has shown that the 15 *F. verticillioides* PKSs represent a significant amount of the diversity within fungal PKSs (Kroken et al. 2003). Analysis of *F. verticillioides PKS4* indicates that it is the homologue of the *F. fujikuroi pks4* gene as they share over 90% identity over more than 2000 amino acids. *PKS4* encodes a PKS required for the synthesis of the bikaverin, a red multicyclic pigment produced by some *Fusarium* spp. including *F. verticillioides* (Linnemannstons et al. 2002).

Examination of the Broad Institutes *F. verticillioides* genome sequence led to the identification of 40 kb DNA sequence flanking the *PKS4* gene. This genomic sequence was then used to BLAST against the FvGI to identify associated TCs/ESTs. We identified three TCs within 21 kb 5′ of *PKS4* and five TCs and two ESTs within 20 kb 3′ of *PKS4* (Figure 1). We then analysed the expression of *PKS4* and flanking genes on the microarray to determine if there were similar patterns of expression from 12 to 120 h growth in GYAM. We found that five of the genes in close proximity to *PKS4* have a similar pattern of expression (Figure 1 and Table 3). Examination of the Broad Institute's gene call set identified a number of additional putative genes located in the region and interspersed among the TCs/ESTs (Figure 1 and Table 3). Because these putative genes were not included in FvGI, they are not represented on the microarray and thus changes in their expression were not measured. Based on chemical arguments (see below) and a shared pattern of expression with *PKS4*, we propose that these six genes comprise the bikaverin gene cluster and that they are involved in bikaverin biosynthesis. Hereafter we refer to these genes as *BIK1* (formerly *PKS4*), *BIK2* to *BIK6*.

Figure 2 depicts a hypothetical polyketide carbon skeleton or backbone for bikaverin and a proposed biosynthetic pathway. A comparison of a possible intermediate released from the PKS to bikaverin indicates four major structural differences: a hydroxyl group at C-15, a keto group at C-17 and methoxy groups at C-4 and C-14. Two of the co-regulated genes flanking *BIK1* have similarity to genes encoding enzymes which could catalyse the synthesis of these constituents. The monooxygenase predicted to be encoded by *BIK2* could oxidize C-17 generating a hydroxyl group and C-15 generating a keto group. The methyltransferase predicted to be encoded by *BIK3* could acetylate the hydroxyl group on both C-4 and C-14. The predicted function of the co-regulated gene *BIK5* is a Zn(II)2Cys6 DNA binding transcription factor which could play a role in regulating transcription of all the cluster genes while the predicted function of *BIK6* is an MFS multidrug transporter which could transport bikaverin out of the fungus.

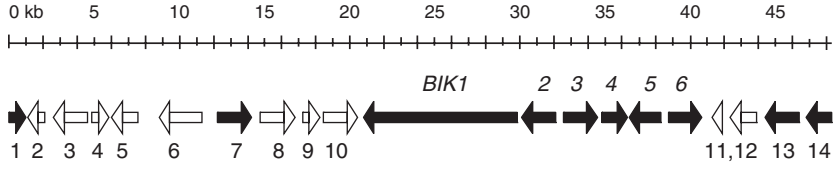

Figure 1. Proposed bikaverin gene cluster and flanking genes. Each bar represents a single gene. The arrows represent the direction of transcription. Solid bars represent genes with sequence identifications on the microarray. Open bars represent genes identified by the Broad Institute's gene annotation system. The six genes (*BIK1–6*) of the bikaverin gene cluster are indicated.

Table 3. Putative PKS4 gene cluster.

Gene[a]	TC/EST[b]	Broad designation[c]	Putative function	$\Delta\log_2$[d]
1	TC32914	FVEG_03369	Unknown	0.03
2		FVEG_03370	Unknown	n.a.
3		FVEG_03371	Heterokaryon incompatibility protein (HET)[f]	n.a.
4		FVEG_03372	Unknown	n.a.
5		FVEG_03373	HET	n.a.
6		FVEG_03374	Hydantoinase/oxoprolinase	n.a.
7	TC31832 TC28546[e]	FVEG_03375	Nucleoside permease	0.17
8		FVEG_03376	HET	n.a.
9		FVEG_03377	Unknown	n.a.
10		FVEG_03378	Serine protease	n.a.
BIK1	TC23604	FVEG_03379	PKS	0.41
BIK2	TC26998	FVEG_03380	Monooxygenase	0.69
BIK3	TC26531	FVEG_03381	Methyltransferase	0.70
BIK4	TC26166	FVEG_03383	Unknown	0.49
BIK5	TC26167	FVEG_03382	Zn(II)2Cys6 transcription factor	0.55
BIK6	TC26182	FVEG_03384	MFS multidrug transporter	0.72
11		FVEG_03385	Unknown	n.a.
12		FVEG_03386	Serine protease	n.a.
13	FVLA077TV	FVEG_03387	Unknown	−0.04
14	FVICP11TH	FVEG_03388	Unknown	0.15

Notes: [a] Gene refers to putative gene position in Figure 1.
[b] TC/EST, tentative consensus sequence/a singleton sequence in the *F. verticillioides* Gene Index.
[c] Broad Institute gene designation released 11 January 2007 based on automated annotation.
[d] $\Delta\log_2$ is the ratio of expression at 120 versus 12 h in liquid GYAM medium in the logarithmic base 2 ratio. n.a., Not available.
[e] These two TCs correspond to the same gene as three of the four clones from which ESTs were derived for TC31832 are the same as TC28546.
[f] HET family proteins contain a conserved region of approximately 150 residues.

The role *BIK4*, the last co-regulated gene, may play in bikaverin synthesis cannot be proposed as a function has not yet been assigned.

Differential expression of alternative splice forms

We have also used the microarray data to examine the relative expression of alternative splice forms (ASFs) of *FUM21*. *FUM21* encodes a Zn(II)2Cys6 transcription factor that is required for the transcriptional regulation of genes in the fumonisin biosynthetic gene cluster and includes 8 introns (Brown et al. 2007). Alignment of available nucleotide sequence of 13 different *FUM21* cDNAs led to the identification of four ASFs (Figure 3). All identified introns in four of the partially sequenced cDNAs were excised. In contrast, one to three introns in nine cDNAs were either not excised or were excised using an alternative 3′ splice site. ASFa, represented by four cDNAs, has an alternative 3′ splice site in the first intron resulting in nine additional nucleotides in the second exon as compared with the functional transcript. ASFb, represented by three cDNAs, retains the second intron. ASFc, represented by a single cDNA, retains the fifth intron while the other seven introns were excised. ASFd, also represented by a single cDNA, retains the third, fourth, and seventh introns while the fifth, sixth and eighth introns were excised.

Translation of any of the *FUM21* ASFs described above would result in different truncated proteins. For ASFa, the first codon at the splice junction is a stop codon and translation would generate a 39 AA protein. Retention of the second intron (ASFb) shifts the open reading frame and translation would generate a 73 AA protein. In either case, the Zn(II)2Cys6 DNA binding domain would be incomplete. Retention of the fifth intron (ASFc) would result in a frameshift and translation would generate a 368 AA protein. Retention of the third intron (ASFd) would also result in a frameshift and translation would generate a 261 AA protein. The putative protein generated by ASFc would have a complete Zn(II)2Cys6 DNA binding domain and a complete fungal transcription factor domain (pfam04082.7). In contrast, the putative protein generated by ASFd, the latter domain would be incomplete. We are currently in the process of determining if any of these truncated proteins serve a function in *F. verticillioides*.

To investigate the role ASFs may play in fumonisin biosynthesis, we examined the expression of transcripts that retain introns 2, 3, 4 or 7 by microarray analysis (Figure 4). Changes in total *FUM21* expression was measured by the average of three different probe sets

(or Sequence IDs) tiled across the entire predicted exon sequence. Figure 4 shows the $\log_2$ of the ratio of expression at 96 versus 12 h; '+' vertical values indicate that expression of a given transcript was greater at 96 than at 12 h, and '−' vertical values indicate expression was less at 96 than at 12 h. Overall expression of *FUM21* transcripts increased over three-fold from 12 to 96 h while expression of transcripts retaining the second intron decreased over two-fold and transcript retaining the seventh intron increased over two-fold. The expression of transcript retaining the third and fourth intron did not change over the same time period.

The different differential expression of transcripts retaining the second intron (I2) versus the seventh intron (I7) suggests a number of possible roles for the *FUM21* ASFs. The decrease in I2 may represent an increasing requirement for a functional *FUM21* protein and the early, higher levels of I2 observed may reflect efforts to limit *FUM21* protein accumulation and resulting subsequent transcription from *FUM* genes. The increase in I7 over time may reflect the requirement for the synthesis of a truncated FUM21p (FUM21I7p; 445 AAs) with a function different from

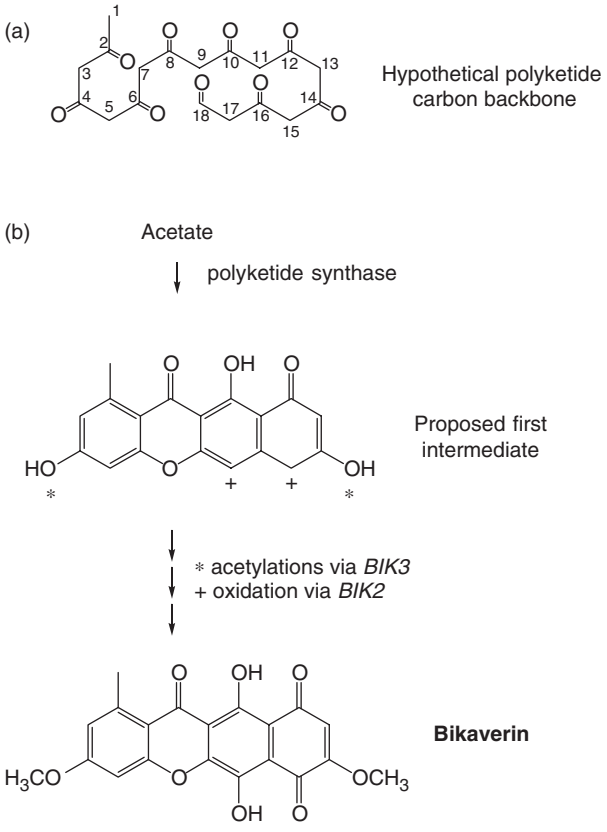

Figure 2. Bikaverin biosynthesis. (a) Hypothetical bikaverin polyketide carbon skeleton or backbone. (b) Proposed biosynthesis of bikaverin from acetate. The '*' below the C-4 and C-14 hydroxyl groups of the proposed intermediate released from the polyketide synthase encoded by *BIK1* indicate two proposed acetylations by the putative methyltransferase encoded by *BIK3*. The '+' below C-15 and C-17 indicate two proposed oxidations by the putative monooxygenase encoded by *BIK2*.

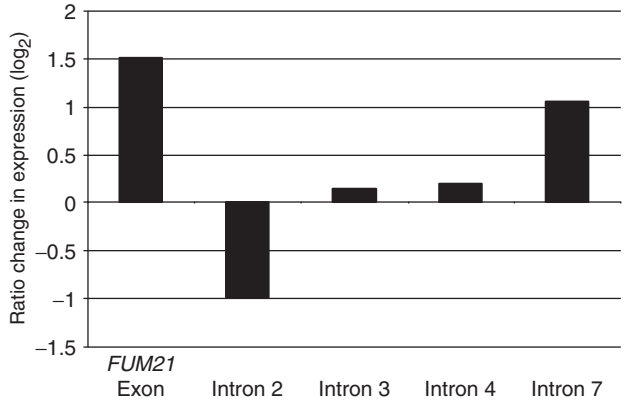

Figure 4. Microarray analysis of *FUM21* expression. *FUM21* expression of the predicted full length transcript was measured by three different probe sets. Relative expression change at 96 h versus 12 h in liquid GYAM medium for *FUM21*, and *FUM21* ASFs retaining introns 2–4 and 7 is indicated on the vertical axis in the logarithmic base 2 ratio.

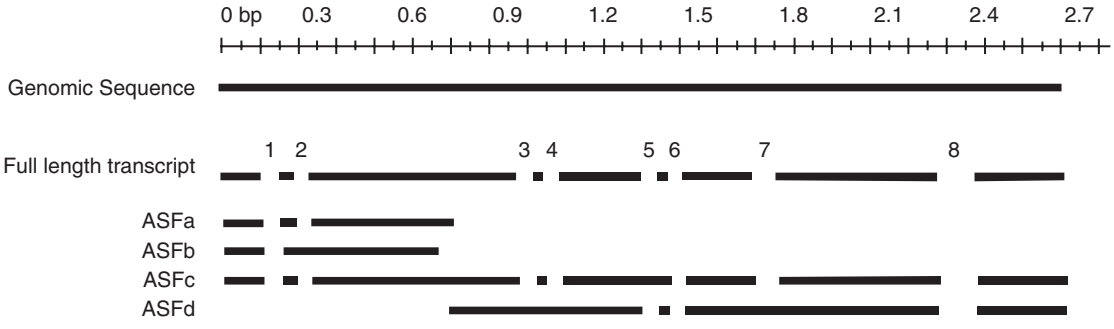

Figure 3. *FUM21* alternative splices. The first bar under the base pair scale represents genomic sequence and the second bar represents the full-length predicted *FUM21* transcript where each numbered gap represents an intron. Translation of this full-length transcript would yield a 672-amino acid protein. ASFa–d represent alternative splice forms of the *FUM21* transcript.

the full length FUM21p (672 AAs). Alternatively, the failure to excise I7 may eliminate transcripts by shunting them to the nonsense-mediated mRNA degradation pathway thereby decreasing the overall *FUM21* transcript level. We are continuing to explore the role of *FUM21* ASFs in fumonisin biosynthesis by examining the effect of proteins resulting from translation of ASF transcripts on *FUM* gene expression and fumonisin production.

Summary

The development and general availability of *F. verticillioides* genomic data to the research community is having a tremendous impact on progress in understanding the regulation of fumonisin biosynthesis. These resources have provided powerful tools to speed the process of identifying genes that are likely to be involved in fumonisin biosynthesis or plant pathogenesis. Understanding these processes will contribute to the ultimate goal of mycotoxin research, namely the elimination or reduction of mycotoxins in food and feed and concomitant reduction of their harmful effects on human and animal health.

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